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Novel *Cryptosporidium* Genotype in Wild Australian Mice (*Mus domesticus*)▽

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A total of 250 mouse fecal specimens collected from crop farms in Queensland, Australia, were screened for the presence of *Cryptosporidium* spp. using PCR. Of these, 19 positives were detected and characterized at a number of loci, including the 18S rRNA gene, the acetyl coenzyme A gene, and the actin gene. Sequence and phylogenetic analyses identified two genotypes: mouse genotype I and a novel genotype (mouse genotype II), which is likely to be a valid species. *Cryptosporidium parvum*, which is zoonotic, was not detected. The results of the study indicate that wild Australian mice that are not in close contact with livestock are probably not an important reservoir of *Cryptosporidium* infection for humans and other animals.

Cryptosporidium sp. is a ubiquitous parasite that is common among wild animals (20). There have been few studies conducted on the epidemiology of *Cryptosporidium* in wild mice; however, it is important to understand if mice are a reservoir of infection for humans and animals. Studies in different geographical areas have reported prevalence rates ranging from 5.0% to 39.2% in wild rodents, but most have relied on morphology for identification (2–4, 20–22).

The limited molecular characterization studies that have been conducted on rodents have identified eight species/genotypes: (i) mouse genotype I, which has been identified in mice and rats and in prairie bison in Spain, Portugal, the United Kingdom, Australia, and the United States (1, 16; L. Xiao et al., unpublished data) and to date has not been identified in humans; (ii) the zoonotic *Cryptosporidium parvum*, which was detected in mice trapped near sheep grazing pastures in Victoria, Australia (16); (iii) *Cryptosporidium muris*, which infects a range of rodents and is generally not zoonotic but which can infect other hosts, including dogs, hamsters, guinea pigs, chipmunks, rabbits, lambs, cats, and humans (4, 9, 10, 11, 18, 19); (iv) a novel genotype detected in one wood mouse (*Apodemus sylvaticus*) sample from the Czech Republic (8); (v) *Cryptosporidium meleagridis*, isolated from a brown rat (*Rattus norvegicus*) in Japan (isolate BR5) (13); (vi) eight isolates from brown rats in Japan that clustered with sequences denoted W19 and W19 variants found in New York storm water (12, 13); (vii) three isolates from brown rats in Japan identical to recently described snake isolate 2162 (AY268584) (13, 25); and (viii) another isolate from brown rats in Japan which showed 100% identity with isolates from nonhuman primates in Sri Lanka (EF446679) (5, 13).

These studies indicate that the majority of wild mice characterized to date do not carry zoonotic species of *Cryptosporidium*. The purpose of the present study was to collect and analyze fecal samples collected from mice trapped from crop farms and grazed pastures in order to determine if only host-adapted genotypes would be detected in the mice from the crop farms.

MATERIALS AND METHODS

Fecal sample collection and DNA extraction. Trapping and collection of the fecal samples were performed on the Darling Downs, which is approximately 200 km west of Brisbane and the Biloela/Moura area of central Queensland, Australia. Traps were set in a variety of agricultural habitats: growing crops, fallows, grazed and ungrazed pastures, and roadside verges. Genomic DNA was extracted from approximately 200 mg of fecal sample using a QIAamp DNA stool kit (Qiagen, Hilden, Germany).

Screening by PCR amplification at the 18S rRNA, actin, and acetyl-CoA loci. All 250 fecal samples were screened for the presence of *Cryptosporidium* at the actin and 18S rRNA loci using a two-step nested PCR as previously described (17, 19). The fecal samples were also screened at the acetyl coenzyme A (acetyl-CoA) synthetase gene using primers that specifically amplify *C. parvum*, *Cryptosporidium hominis*, and mouse genotype I. A primary product of ~820 bp was amplified using the forward primer ACoAF2 (5′-GAA TAG GAG CTG TAC ATA TGG-3′) and reverse primer ACoAR2 (5′-CTA TAG AAC ATC TCT CTT CAC C-3′). Each 25-μl PCR mixture consisted of 1× DNA polymerase reaction buffer (Fisher Biotechnology), 1.5 mM $MgCl_2$ (Fisher Biotechnology), 200 μM deoxynucleoside triphosphate (Promega), 0.5 U of *Tth* plus DNA polymerase (Fisher Biotech), and 12.5 pmol of forward and reverse primers. The PCR cycles were performed using an Applied Biosystems Gene Amp PCR system 2700 thermocycler with 45 cycles of 94°C for 30 s, 60°C for 20 s, and 72°C for 45 s. For the secondary PCR, an ~347-bp fragment was amplified using 1 μl of the primary product together with the internal forward primer ACoAF1 (5′-GGA CCT ATT GAA TTT GTC AAG G-3′) and internal reverse primer ACoAR1 (5′-GAG TAA TTC TGT GTC TCT CCA C-3′). The PCR mixture and thermocycler conditions were identical to those for the primary reaction.

Sequence and phylogenetic analyses. PCR products were purified as previously described (17) and sequenced using an ABI Prism Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Nucleotide sequences were analyzed using Chromas Lite version 2.0 (http://www.technelysium.com.au) and aligned using Clustal W (http://clustalw.genome.jp). Distance estimation was conducted using TREECON (23), based on evolutionary distances calculated with the Tamura-Nei model and grouped using neighbor joining. Parsimony analysis was conducted using MEGA version 3.1 (14). The confidence of groupings was assessed by bootstrapping, using 1,000 replicates. In construction of the neighbor-joining and maximum parsimony trees, sequences of *Eimeria bovis* (GenBank accession no. EBU77084) and *Toxoplasma gondii* (U104429) were used as outgroups for the 18S and actin loci. Phylogenetic trees were not constructed for the acetyl-CoA locus due to the lack of

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TABLE 1. Summary of genotypes and trapping locations for *Cryptosporidium*-positive mice

Date of collection (day/mo/yr)	Location	Agricultural description	Isolate code	*Cryptosporidium* genotype
03/06/2005	Northern Downs #3	Sorghum stubble	S7	Mouse genotype II
	Northern Downs #3	Sorghum stubble	S8	Mouse genotype I
	Northern Downs #3	Sorghum stubble	S12	Mouse genotype II
	Northern Downs #3	Sorghum stubble	S15	ND[a]
	Northern Downs #5	Sorghum mulch	S21	Mouse genotype II
	Northern Downs #12	Unmown road verge	S24	Mouse genotype II
	Northern Downs #7	Railway line, long ungrazed grass and weeds	S25	Mouse genotype II
07/02/2005	Curraweena (Darling Downs)	Barley stubble	S45	Mouse genotype II
	Akeringa (Darling Downs)	Mature sorghum	S49	Mouse genotype I
13/7/2005	Akeringa (Darling Downs)	Sorghum stubble	S73	Mouse genotype I
	Akeringa (Darling Downs)	Sorghum stubble	S77	ND
	Akeringa (Darling Downs)	Sorghum stubble	S78	Mouse genotype II
21/7/2005	Bungarrie (Darling Downs)	Sorghum stubble	S103	Mouse genotype II
	Bungarrie (Darling Downs)	Sorghum stubble	S110	Mouse genotype II
31/8/2005	Oakleigh Park (Darling Downs)	Mature millet	S126	Mouse genotype I
	Oakleigh Park (Darling Downs)	Mature millet	S129	Mouse genotype I
15/9/2005	Callide Valley (Central Queensland)	Fallow cereal paddock	S152	Mouse genotype I
	Callide Valley (Central Queensland)	Barley stubble	S157	Mouse genotype II
	Callide Valley (Central Queensland)	Cropped barley field	S163	Mouse genotype II

[a] ND, not determined.

available sequences for *Cryptosporidium* species and genotypes other than *C. parvum* and *C. hominis* at this locus.

Morphometric analysis. Isolates that were positive by PCR were also checked by microscopy to determine the size of the oocysts. Microscopy for *Cryptosporidium* was carried out using malachite green negative staining (6). Oocysts were measured using the Optimus Image analysis package version 5.2 at ×1,000 magnification.

Statistical analysis. A chi-square test for independence was used to measure the association between obtaining a positive result and/or genotype versus age (adult/juvenile) and gender (female/male). An analysis of variance was performed to determine if there was an association between length and weight of mice with the presence of *Cryptosporidium* and the genotype. These analyses were conducted using the computer package SPSS version 14.0.0.

Nucleotide sequence accession numbers. The unique partial 18S rRNA and actin sequences generated as part of this study have been deposited in the GenBank database under accession numbers EF546483 and EF546484.

RESULTS

***Cryptosporidium* spp. in feces of wild mice.** Of the 250 fecal samples which were collected from trapped wild mice (*Mus domesticus*), a total of 19 positives were detected by PCR screening, giving a prevalence rate of 7.6% (95% confidence interval, 4.3 to 10.9). As difficulties were initially experienced in amplifying mouse genotype I at the 18S rRNA locus, samples were also screened at the actin locus as well as the acetyl-CoA gene locus, as the acetyl-CoA locus is specific for mouse genotype I, *C. parvum* and *C. hominis*. Sequences were obtained for 17 of the 19 positives. Two distinct genotypes were identified: 7 were the mouse genotype, and 10 were a novel genotype (mouse genotype II) which has not been previously described. *Cryptosporidium hominis, C. parvum*, and *C. muris* were not detected in any of the samples (Table 1).

Morphometric analysis. Attempts were made to conduct morphometric measurements on mouse genotype II; however, as oocysts were present in very low numbers, it was not possible to conduct measurements on sufficient numbers of oocysts. The measurements that were made, however, indicated that the oocyst size did not differ significantly from *C. parvum* (5.2 by 4.3 μm) (7).

Statistical analysis. Statistical analysis was conducted to determine the association between *Cryptosporidium* spp. versus the age, sex, length, and weight of the mice trapped. The analysis showed that there was no association between *Cryptosporidium* spp. versus the age, sex, length, and weight of the mice (data not shown).

Genetic relationships among mouse-derived genotypes. Distance- and parsimony-based phylogenetic analyses grouped both mouse genotype I and mouse genotype II with the intestinal *Cryptosporidium* species/genotypes (data not shown). At the 18S locus, both distance and parsimony analyses grouped mouse genotype II most closely with *Cryptosporidium suis* and a novel *C. suis*-like genotype (K4515) recently identified in cattle (15) (Fig. 1a). The genetic similarity between mouse genotype II and *C. suis*/K4515 was 99.6%, and mouse genotype II shared 99.3% similarity with muskrat genotype II (Table 2). At the actin locus, mouse genotype II again grouped most closely with *C. suis* (Fig. 1b) and exhibited 94% similarity with *C. suis* and 92.1% similarity with its next closest relative, the deer mouse genotype (Table 2).

DISCUSSION

In the present study, two genotypes, mouse genotype I and mouse genotype II, were identified in mice from the Darling Downs and central Queensland, Australia. The prevalence rate of 7.6% (95% confidence interval, 4.3 to 10.9) is within the

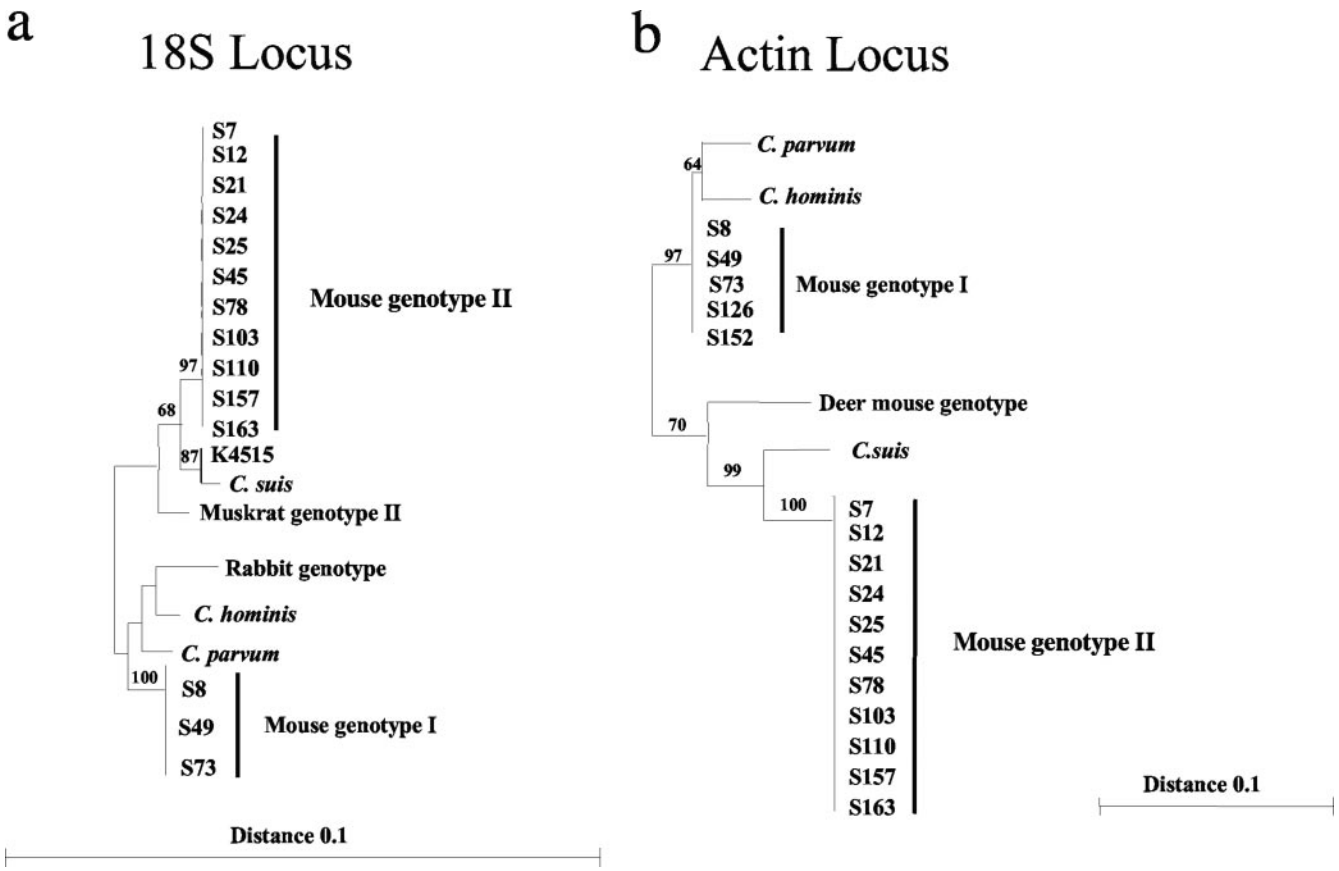

FIG. 1. Evolutionary relationships of *Cryptosporidium* mouse genotype I and II isolates inferred by neighbor-joining analysis of Kimura distances calculated from pairwise comparison of 18S rRNA (a) and actin (b) sequences. The percent bootstrap support (>60%) from 1,000 pseudoreplicates is indicated at the left of the supported node.

range of previous studies (5.0% to 39.2%) (2–4, 20–22). A previous study which examined mice from different geographical locations, including Australia, the United Kingdom, and Spain, revealed that the majority of *Cryptosporidium*-positive mice were infected with mouse genotype I (16). Mouse genotype II was not detected in that study, but this may have been due to the fact that screening was done using genotype-specific acetyl-CoA primers and 18S rRNA primers designed against the limited range of *Cryptosporidium* sequences that were available at the time, and these may have been unable to amplify mouse genotype II. In that study, five of the Australian mouse samples were infected with the zoonotic *C. parvum*; these mice were trapped on farms in Victoria where large numbers of sheep were grazing. This indicates that sheep may transmit *C. parvum* to mice, which may in turn transmit *Cryptosporidium* to other domestic animals. In the present study, the zoonotic *C. parvum* was not detected. This may be because all the positive samples were from crop farms where the mice were unlikely to encounter sheep and cattle. It is possible that mice are only occasionally infected with *C. parvum* during periods of heavy environmental contamination.

TABLE 2. Similarities of mouse genotypes I and II to their closest relatives and between currently accepted species at the 18S rRNA and actin loci

Comparison	% Similarity at locus	
	18S rRNA	Actin
All valid species of *Cryptosporidium* (range of similarities)	89–99.8	76.0–98.7
Mouse genotype II vs valid species of *Cryptosporidium*	90.6–99.6	79.1–94
Mouse genotype I vs *C. hominis*	99.8	98.7
Mouse genotype I vs *C. parvum*	99.8	99.2
Mouse genotype II vs *C. hominis*	99.1	88.8
Mouse genotype II vs *C. parvum*	98.9	88.5
Mouse genotype II vs *C. suis*	99.6	94
Mouse genotype II vs muskrat genotype II	99.3	ND[a]
Mouse genotype II vs deer mouse genotype	97.2	91.2

[a] ND, not determined.

Phylogenetic analyses at both the 18S rRNA and actin loci suggest that mouse genotype II is most closely related to *C. suis* and may be a distinct species, as the range of genetic similarities between mouse genotype II and all other *Cryptosporidium* species at the 18S rRNA locus was 90.6 to 99.6% and at the actin locus it was 79.1 to 94%. This is within the range of the percent similarities between currently accepted *Cryptosporidium* species at the 18S rRNA locus (89 to 99.8%) and the actin locus (76 to 98.7%) and is one of the criteria used to delimit species within the genus *Cryptosporidium* (24).

Mouse genotype II also appears to be host specific, as it has

been found only in mice and has never been reported in humans. A previous study by Annika Boxell (unpublished) detected mouse genotype II in wild mice from Macquarie Island (located approximately halfway between Australia and Antarctica) and Thevenard Island (located off the coast of Western Australia). *Cryptosporidium* was detected in 34.8% of fecal samples from mice (23/66) collected from these islands, and of these, 26% (6/23) were 100% identical to mouse genotype II at the 18S rRNA locus. Another study described a novel genotype from one wood mouse from the Czech Republic which was most closely related to *C. suis* (8). Unfortunately, it is not possible to determine if the novel genotype in that study is the same as mouse genotype II identified as part of the present study, as only the *Cryptosporidium* oocyst wall protein and not the 18S or actin locus was sequenced. The rat-derived 18S rRNA *Cryptosporidium* sequences from a recent study in Japan (13) were not included in the phylogenetic analysis in the present study, as those were considerably shorter. However, sequence alignments revealed that none of these isolates matched mouse genotype I or mouse genotype II. More studies need to be conducted to confirm the host and geographic ranges of mouse genotype II.

The results of this study indicate that mice that are not in close contact with livestock are most commonly infected with two genetically distinct and host-adapted genotypes of *Cryptosporidium*: mouse genotype I and the novel mouse genotype II. The potential for mouse genotypes I and II to cause disease in mice or humans is unknown, but the fact that mouse genotypes I and II have never been reported in humans suggests that they are unlikely to be of public health significance. Further studies are required to understand the extent of host adaptation for mouse genotypes I and II.

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